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Exploring the venom of the forest cobra snake: Toxicovenomics and antivenom profiling of *Naja melanoleuca*

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Abstract

A toxicovenomic analysis of the venom of the forest cobra, *N*. *melanoleuca*, was performed, revealing the presence of a total of 52 proteins by proteomics analysis. The most abundant proteins belong to the three-finger toxins (3FTx) (57.1 w%), which includes post-synaptically acting α-neurotoxins. Phospholipases A_2 (PLA_2) were the second most abundant group of proteins (12.9 w%), followed by metalloproteinases (SVMPs) (9.7 w%), cysteine-rich secretory proteins (CRISPs) (7.6 w%), and Kunitz-type serine proteinase inhibitors (3.8 w%). A number of additional protein families comprised each less than 3 w% of venom proteins. A toxicity screening of the fractions, using the mouse lethality test, identified toxicity in RP-HPLC peaks 3, 4, 5 and 8, all of them containing α-neurotoxins of the 3FTx family, whereas the rest of the fractions did not show toxicity at a dose of 0.53 mg/kg. Three polyspecific antivenoms manufactured in South Africa and India were tested for their immunoreactivity against crude venom and fractions of *N*. *melanoleuca*. Overall, antivenoms immunorecognized all fractions in the venom, the South African antivenom showing a higher titer against the neurotoxin-containing fractions. This toxicovenomic study identified the 3FTx group of α-neurotoxins in the venom of *N*. *melanoleuca* as the relevant targets to be neutralized.

(200 words)

Biological significance

A toxicovenomic analysis of the venom of the forest cobra, also known as black cobra, *Naja melanoleuca*, was performed. Envenomings by this elapid species are characterized by a progressive descending paralysis which starts with palpebral ptosis and, in severe cases, ends up with respiratory arrest and death. A total of 52 different proteins were identified in this venom. The most abundant protein family was the three-finger toxin (3FTx) family, which comprises almost 57.1 w% of the venom, followed by phospholipases A_2 (PLA_2) (12.9 w%). In addition, several other protein families were identified in a much lower percentage in the venom. A toxicity screening of the fractions, using the mouse lethality assay, identified four peaks as those having toxicity higher than that of the crude venom. These fractions predominantly contain α-neurotoxins of the 3FTx family. This toxicovenomic characterization agrees with the clinical and experimental manifestations of envenomings by this species, in which a strong neurotoxic effect predominates. Therefore, our findings suggest that immunotherapy against envenomings by *N. melanoleuca* should be directed towards the neutralization of 3FTxs; this has implications for the improvement of current antivenoms and for the development of novel antivenoms based on biotechnological approaches. A screening of the immunoreactivity of three antivenoms being distributed in sub-Saharan Africa revealed that they immunoreact with the fractions containing α-neurotoxins, although with different antibody titers.

1. Introduction

The forest cobra, also known as the black cobra (*Naja melanoleuca*), is a highly venomous member of the elapid snake family, reaching up to 3.1 meters in length, and being able to deliver venom yields above 1 gram per milking [1]. *N. melanoleuca* is the largest of the African cobra species and it is known to inhabit moist river areas, primary and secondary forests, and suburban habitats in Western, Central, and Southern Africa [2–4]. Its coloration may vary between three different color morphs, and it is active during the day, where it feeds on mammals, frogs, and fish [2,3]. From the clinical standpoint, envenomings by *N. melanoleuca* have been classified within the syndromic category 3, characterized by progressive paralysis (neurotoxicity) [5]. Patients develop a descending progressive paralysis which starts with ptosis, external ophtalmoplegia and weakness of muscles innervated by the cranial nerves, with patients having difficulties in swallowing and speaking. Eventually the respiratory muscles become paralyzed, and death ensues unless mechanical ventilation is provided [5].

Currently, six antivenoms are claimed to be effective against envenomings from *N. melanoleuca* [6]. Due to the severity of envenomings, *N. melanoleuca* is classified by the WHO as a category 1 snake of highest medical importance (http://apps.who.int/bloodproducts/snakeantivenoms/database/). Therefore, it is of high relevance to obtain a deep understanding of the composition of *N. melanoleuca* venom. To this date, no quantitative venom proteome has been reported for *N. melanoleuca*, however, several biochemical studies have reported that the venom contains long and short neurotoxins [7,8], cytotoxins [9–11], phospholipases A_2 [12,13], and 'weak' toxins [9,14].

In order to develop safe and effective antivenoms that can protect against envenoming from *N. melanoleuca*, it is not only important to know the venom composition. It is also essential to understand which toxins are the medically most relevant to target. For this purpose, the combination of venomics and the Toxicity Score [15] may be employed to unveil which toxins are the main culprits responsible for the clinical manifestations of *N. melanoleuca* envenomings. Being able to identify these key toxins may not only help guide traditional antivenom development, but may also aid rational antitoxin discovery approaches based on biotechnology [16].

Here, we report the first toxicovenomics study of the venom of *N. melanoleuca*, providing a quantitative estimation of its proteome alongside an assessment of the medical importance of the individual venom fractions and an evaluation of the immunorecognition pattern of three antivenoms in use in sub-Saharan Africa.

2. Materials and Methods

2.1 Snake venom

Venom of *N. melanoleuca* was obtained from Latoxan SAS, Valence, France, from a pool of 7 specimens collected in Uganda. Venoms from *N. nigricollis* and *N. mossambica* used for comparison in *in vitro* enzymatic assays were also obtained from Latoxan from pools of several specimens collected in Tanzania. Venom from *Bothrops asper* was obtained as a pool from several specimens from Costa Rica kept at Instituto Clodomiro Picado, Universidad de Costa Rica, Costa Rica.

2.2 Venom separation by reverse-phase HPLC and SDS-PAGE

Following the 'snake venomics' analytical strategy, crude venom was

fractionated involving a combination of RP-HPLC and SDS-PAGE separation [17]. Two mg of venom was dissolved in 200 µL of water containing 0.1% trifluoroacetic acid (TFA; solution A) and separated by RP-HPLC (Agilent 1200) on a C_{18} column (250 x 4.6 mm, 5 µm particle; Supelco). Elution was carried out at 1 mL/min by applying a gradient towards solution B (acetonitrile, containing 0.1% TFA): 0% B for 5 min, 0–15% B over 10 min, 15–45% B over 60 min, 45–70% B over 10 min, and 70% B over 9 min, as previously described [18]. Fractions were collected manually, dried in a vacuum centrifuge, redissolved in water, reduced with 5% β-mercaptoethanol at 100°C for 5 min, and further separated by SDS-PAGE in 15% gels. Colloidal Coomassie blue G-250 was used for proteins staining, and a ChemiDoc® recorder and ImageLab® software (Bio-Rad) were used to acquire gel images.

2.3 Protein identification by tandem mass spectrometry of tryptic peptides

From the polyacrylamide gels protein bands were excised and subjected to reduction (10 mM dithiothreitol), alkylation (50 mM iodoacetamide), and overnight in-gel digestion with sequencing grade trypsin (Sigma), in 50 mM ammonium bicarbonate at 37 °C. Tryptic peptides were extracted with 50% acetonitrile containing 1% TFA, and analyzed by MALDI-TOF-TOF on an AB4800-Plus Proteomics Analyzer (Applied Biosystems). Digested and extracted peptides were mixed with an equal volume of saturated α-cyano-hydroxycinnamic acid (in 50% acetonitrile, 0.1% TFA), and spotted (1 µL) onto an Opti-TOF 384-well plate, dried, and analyzed in positive reflector mode. TOF spectra were acquired using 500 shots at a laser intensity of 3000. TOF/TOF fragmentation spectra were acquired using 500 shots at a laser intensity of 3900 for the automatically selected ten most intense

precursor ions. CalMix® standards (ABSciex) spotted onto the same plate were used for external calibration in each run. Resulting spectra were searched against the UniProt/SwissProt database for Serpentes (20150217) using ProteinPilot® v.4 and the Paragon® algorithm (ABSciex) at ≥ 95% confidence, or, in few cases, manually interpreted, and the deduced sequences searched using BLAST (http://blast.ncbi.nlm.nih.gov) for assignment of protein family by similarity.

2.4 Relative protein abundance estimations

The relative abundance of the venom proteins was estimated using the ChemStation® software (Agilent) to integrate the areas of their chromatographic peaks at a wavelength of 215 nm, roughly corresponding to peptide bond abundance [17]. When HPLC peaks contained several electrophoretic bands, ImageLab® (Bio-Rad) was used to assign their percentage distributions by densitometry. Finally, for electrophoretic bands containing more than one protein according to MALDI-TOF-TOF analysis, their percentage distributions were estimated based on the corresponding intensities of the intact protein ions, as observed in the nESI-MS analysis. For this, a 10 μL sample of the HPLC fraction was loaded into a metal-coated capillary (Proxeon) and directly infused into a nano-spray source of a QTrap 3200 mass spectrometer (Applied Biosystems) operated at 1300 V in enhanced multi-charge mode. Deconvolution of spectra was performed with the aid of the Bayesian protein reconstruction tool of Analyst v.1.5. Intensities lower than 5% (relative to the major protein ions in these mixtures) were considered as traces. Protein abundances were calculated on the basis of protein content percentage (w%).

2.5 In vitro enzymatic activities

2.5.1. Phospholipase A_2 activity

PLA_2 activity was assayed using the monodisperse synthetic chromogenic substrate 4-nitro-3-octanoyloxybenzoic acid (NOBA) [19]. 25 µL of solution, containing various amounts of venom, were mixed with 200 µL of 10 mM Tris, 10 mM $CaCl_2$, 0.1 M NaCl, pH 8.0, and 25 µL of NOBA to achieve a substrate concentration of 0.32 mM. Plates were incubated at 37 °C for 60 min, and absorbances were recorded at 405 nm (Multiskan FC, Thermo Scientific). For comparative purposes, the activities of the venoms of *N. mossambica*, *N. nigricollis*, and the viperid snake *Bothrops asper* were also assessed.

2.5.2 Proteinase activity

Proteinase activity was assayed by adding 20 µg of venom to 100 µL of azocasein (10 mg/mL in 50 mM Tris–HCl, 0.15 M NaCl, 5 mM $CaCl_2$ buffer, pH 8.0), and the mixture was incubated for 90 min at 37 °C. The reaction was terminated by addition of 200 µL of 5% trichloroacetic acid. After centrifugation (5 min, 6,000 × g), 150 µL of supernatants were mixed with 100 µL of 0.5 M NaOH, and absorbances were recorded at 450 nm (Multiskan FC, Thermo Scientific). The absorbance of azocasein incubated with distilled water alone was used as a blank, being subtracted from all readings [20]. For comparative purposes, the activities of the venoms of *N. mossambica*, *N. nigricollis*, and *Bothrops asper* were also assessed.

2.6 Toxicological profiling

2.6.1 Animals

In vivo assays were performed in CD-1 mice of both sexes, provided by Instituto Clodomiro Picado, following protocols approved by the Institutional

Committee for the Use and Care of Animals (CICUA), University of Costa Rica. Mice were provided food and water *ad libitum*.

2.6.2 Toxicity of crude venom and isolated venom fractions

The acute toxicity of venom fractions was initially screened by intravenous (i.v.) injection of 10 μg of toxin per mouse (0.53 mg/kg) for all fractions devoid of snake venom metalloproteinases (SVMPs) in groups of three mice (18–20 g body weight). Fractions that were not lethal at this level were not further investigated, whereas precise LD_{50}s were determined in groups of four mice for fractions which did kill mice at this dose, and for the whole venom. Toxicity Scores were calculated according to Laustsen et al. [15], on the basis of the abundance (w%) of each fraction. Additionally, a Molecular Toxicity Score was introduced, which was calculated using the molecular abundance (mol%) of each fraction based on the molecular mass of identified toxins. Various amounts of venom or venom fractions were dissolved in phosphate-buffered saline (PBS; 0.12 M NaCl, 0.04 M sodium phosphate buffer, pH 7.2) and injected in the caudal vein, in a volume of 100 μL. Deaths occurring within 24 h were recorded, and LD_{50}s were calculated by probits [21], using the BioStat® software (AnalySoft).

2.7 Antivenoms

Polyspecific antivenoms from the following manufacturers were employed: (a) SAIMR (South African Institute for Medical Research) Polyvalent Snake Antivenom from South African Vaccine Producers (Pty) Ltd (batch number BC02645, expiry date 07/2016); (b) Snake Venom Antivenom (Central Africa) from VINS Bioproducts

Ltd (batch 12AS13002, expiry date 04/2017); (c) Snake Venom Antivenom (African) from VINS Bioproducts Ltd (batch 13022, expiry date 01/2018).

2.8 Immunoreactivity of antivenoms against crude venom and venom fractions by ELISA

Wells in MaxiSorp™ plates (NUNC, Roskilde, Denmark) were coated with 1 μg of each HPLC venom fraction, or crude venom, dissolved in 100 μL PBS overnight. Next day, the wells were washed three times with PBS and blocked by adding 100 μL PBS containing 2% (w:v) bovine serum albumin (BSA, Sigma), and incubated at room temperature for 1 h. Then, plates were washed five times with PBS. A dilution of each antivenom in PBS + 2% BSA was prepared, and 100 μL of these solutions were added to each well in triplicates and incubated for 2 h. Plates were then washed five times with PBS. Subsequently, 100 μL of a 1:2000 dilution of conjugated antibody (Sigma A6063, rabbit anti-horse IgG (whole molecule)-alkaline phosphatase in PBS + 1% BSA) was then added to each well. Following 2 h of incubation, the wells were washed five times with FALC buffer (0.05 M Tris, 0.15 M NaCl, 20 μM $ZnCl_2$, 1 mM $MgCl_2$, pH 7.4). Color development was achieved by addition of 100 μL *p*-nitrophenyl phosphate (1 mg/mL in 9.7% v/v diethanolamine buffer, pH 9.8) and absorbances at 405 nm were recorded (Multiskan FC, Thermo Scientific).

3.0 Results and Discussion

3.1 Venomics

A bottom-up venomic characterization of *N. melanoleuca* venom was performed. Using RP-HPLC, the venom was resolved into 33 fractions, where the first, second and sixth fractions eluting from the column did not contain proteins as found in SDS-

PAGE. Further resolving by SDS-PAGE of the 30 remaining fractions yielded 63 bands (Figure 2), of which the protein identities for 62 bands were positively identified upon in-gel digestion and MALDI-TOF-TOF analysis. Within these bands a total of 52 different proteins were identified. As described previously [22,23], some fractions contained toxins in both monomer and dimer form, as exemplified by fraction number 10 (see Figure 2). To uncover the overall protein composition of *N. melanoleuca* venom, the identified proteins were assigned to families and expressed as percentages of total protein content (see Figure 3 and Table 1). The most abundant proteins were found to belong to the three-finger toxin (3FTx) family (57.1 w%), followed by proteins from the phospholipase A_2 (PLA_2) family (12.9 w%) (Figure 3). 3FTxs in elapid venoms share a common structural scaffold consisting of 60-80 amino acids, with three β-stranded loops extending from a small, globular, hydrophobic core [24–26]. Despite this highly conserved structure, 3FTxs display a wide range of activities [27], although the main 3FTxs identified in the venom of *N. melanoleuca* are type I and II α-neurotoxins and cytotoxins (see Table 1). Both type I and II α-neurotoxins target the nicotinic acetylcholine receptor at the end-plate of muscle fibers, causing flaccid paralysis in victims and prey, potentially culminating in respiratory failure and death [28–30]. The cytotoxins identified in this study derive from the type IA cytotoxin sub-subfamily. Cobra cytotoxins are amphiphilic toxins known for inducing cellular damage through disruption of the cell membrane, which may lead to tissue necrosis [31,32]. However, experimental and clinical envenomings by *N. melanoleuca* are not characterized by tissue necrosis possibly due to the relatively low content of cytotoxins as compared to the predominant α-neurotoxins.

The second-most abundant toxin family found in the venom of *N. melanoleuca* is the phospholipase A_2 (PLA_2) family (12.9 w%), which is generally found in

abundance in both viperids and elapids and, in the case of elapids, has evolved from pancreatic PLA_2 digestive enzymes [33]. The catalytic function of PLA_2s is to cleave phospholipids at the *sn*-2 position in the glycerol backbone. In snake venoms, however, PLA_2s not only play a role in the digestion of prey, but also exhibit a variety of toxicological effects [34]. As in other elapid venoms, the PLA_2s found in *N. melanoleuca* belong to the Group I, catalytically-active D49 enzymes [33]. In agreement, *N. melanoleuca* venom showed PLA_2 activity, similar to the venom of *N. nigricollis* but lower than that of venoms of *N. mossambica* and *Bothrops asper* (Figure 4A). Despite the presence of 9.7% SVMPs in the venom proteome, very low proteinase activity was observed on azocasein (Figure 4B). It is likely that the SVMPs of *N. melanoleuca* have restricted substrate specificity as observed in other elapid species [22,23]

Other protein families found in lower proportions in the venom of *N. melanoleuca* include cysteine-rich secretory proteins (CRISP; 7.6 w%), Kunitz-type serine proteinase inhibitors (BPTI/Kunitz; 3.8 w%), flavin monoamine oxidases (FMO; 2.5 w%), and traces of nerve growth factors (NGF < 0.4 w%), ohanin/vespryn family (OHA; < 0.5 w%), DNA/RNA endonucleases (DRE < 0.3 w%), endonuclease/phosphodiesterase (PDE; 1.1 w%), glutathione peroxidase family (GPF; 0.9 w%), type-B carboxylesterase/lipase family (CELF; 1.1 w%), and selectins (SUSHI; < 0.3 w%).

3.2. Toxicity of venom fractions

Toxicity testing was carried out for all venom fractions devoid of SVMPs, since the solvents used for RP-HPLC denature these proteinases (Table 2). First, the LD_{50} of crude venom was determined to be 0.66 mg/kg (95% confidence interval 0.49-0.92

mg/kg). This value was higher than previously determined LD_{50}s (i.v. 0.289 mg/kg), reported on http://snakedatabase.org/pages/LD50.php. It is likely that this can be explained by geographical variation in venom toxicity which is known to occur in species having a wide distribution [35]. A cut-off value of 10 µg per mouse (0.53 mg/kg) was chosen for toxicity screening of the venom fractions. From Table 2 it was evident that the majority of fractions inducing lethality within 24 h were those containing type I and II α-neurotoxins. LD_{50}s were determined for fractions that showed lethality at 0.53 mg/kg, more specifically fractions 3, 4, 5, and 8, as depicted in Table 2. These LD_{50}s were lower than the overall LD_{50} of the crude venom suggesting that the toxins present in these fractions are of high medical relevance. According to their Toxicity Scores, the most potent fraction was fraction 8 (TS = 88, Molecular TS = 115.5), containing a homolog of Long neurotoxin OH-55 *Ophiophagus hannah* (Q53B58), Long neurotoxin 2 *Naja melanoleuca* (P01338), and Weak toxin S4C11 *Naja melanoleuca* (P01400). Previous studies have found Weak toxin S4C11 *Naja melanoleuca* (P01400) to have an LD_{50} of 20 mg/kg [9], strongly suggesting that the long neurotoxins are the cause of the high toxicity of this fraction. From the MALDI-TOF-TOF analysis fractions 3, 4, and 5 were found to contain similar α-neurotoxins, with all three fractions having LD_{50}s in the same range. Due to differences in abundances, Toxicity Scores for these fractions differed (TS = 59.2, 4.6, and 10.0, Molecular TS = 78.8, 15.0, and 6.9, respectively). Nevertheless, the similarity in both the sequences obtained (Table 1) and the LD_{50}s (Table 2) suggest that the toxins present in these fractions are likely to be similar isoforms. No other toxins were found to show lethality at the pre-determined cut-off value. Mice injected with fractions 3, 4, 5, and 8 showed evident manifestations of respiratory paralysis, indicating that the cause of death was neurotoxin-induced respiratory arrest.

The toxicity analysis of fractions strongly suggests that other venom components, such as PLA_2s, do not play a central role in the overall toxicity. Despite the fact that neurotoxic PLA_2s occur in many elapid snake venoms [36], and although neurotoxic PLA_2s have been described in some *Naja* sp venoms [37], in general the neurotoxicity of cobra venoms is based on the action of post-synaptically acting 3FTxs. Likewise, other minor components present in *N. melanoleuca* venom do not seem to exert lethal effects either, as judged by our toxicity screening.

According to their Toxicity Scores, the most therapeutically relevant targets of *N. melanoleuca* to be neutralized with antivenom were found to be homologs of Alpha-neurotoxin NTX-1 from *N. sputratix* (Q9YGJ6), Short neurotoxin 1 from *N. melanoleuca* (P01424), Long neurotoxin 2 from *N. melanoleuca* (P01388), and Long neurotoxin OH-55 *O. hannah* (Q53B58) which are α-neurotoxins that bind to the nicotinic acetylcholine receptor, thereby abrogating neuromuscular transmission.

3.3 Immunoprofiling of antivenoms

Three polyspecific antivenoms, distributed in sub-Saharan Africa, were tested for their ability to recognize *N. melanoleuca* crude venom and fractions by ELISA. First, ELISA titration curves were determined against immobilized crude venom with the highest binding found to be VINS African, followed by SAVP, and VINS Central Africa, when normalized according to protein concentration (Figure 5). The immunization mixture of both VINS African and SAVP contain the venom of *N. melanoleuca*, whereas VINS Central Africa solely consists of venom from three species of the Viperidae family and *Dendroaspis polylepis*. Thus, cross-reactivity between similar toxins present in these species and *N. melanoleuca* is likely to exist.

When using solid-phase immunoassays of antivenoms against crude venoms, prediction of cross-reactivity may be of limited value as antibodies tend to bind to the highly immunogenic venom components, which are not always the most medically relevant ones. Therefore, it is important to consider the recognition pattern for antivenoms against individual venom components or fractions. To further investigate these immunorecognition patterns, all three antivenoms were subjected to yet another ELISA assay against the different venom fractions (Figure 6). From these results it was observed that the three antivenoms generally have a similar recognition pattern. However, the SAVP antivenom does appear to have higher antibody titers than the other antivenoms against the medically most relevant fractions containing the α-neurotoxins (fractions 3, 4, 5, and 8) (see Figure 6).

4.0 Concluding remarks and outlook

In the present study, the venom of *N*. *melanoleuca* was, for the first time, subjected to a thorough toxicovenomics analysis. This revealed that the venom was dominated by three-finger toxins (57.1 w% of the venom) and phospholipase A_2s (12.9 w% of the venom), of which particularly the three-finger toxins were determined to be the most toxic fractions of the venom evaluated by their Toxicity Score. Additionally, other protein families (CRISPs, nerve growth factor, Kunitz-type serine protease inhibitor, ohanin/vespryn, SVMPs, DNA/RNA non-specific endonuclease, endonuclease/phosphodiesterase, flavin monoamine oxidase, glutathione peroxidase, Type-B carboxylesterase/lipase, and selectins) were determined to be present in the venom. Immunoprofiling of three antivenoms by ELISA with the different fractions of *N*. *melanoleuca* venom revealed a similar pattern of immunorecognition, although the South African antivenom exhibited slightly higher signals against the

toxicologically relevant neurotoxins. Our toxicovenomic observations indicate that an effective antivenom against the venom of *N*. *melanoleuca* should contain neutralizing antibodies against venom components having homology to the α-neurotoxins Alpha-neurotoxin NTX-1 from *N*. *sputratix* (Q9YGJ6), Short neurotoxin 1 from *N*. *melanoleuca* (P01424), Long neurotoxin 2 from *N*. *melanoleuca* (P01388), and Long neurotoxin OH-55 *O*. *hannah* (Q53B58). Hopefully, these studies may help lay the foundation for developing more efficacious antivenoms, based on traditional or novel biotechnological approaches.

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Figure legends

Figure 1: *Naja melanoleuca* in a raised position displaying its characteristic cobra hood.

Figure 2: Separation of *N. melanoleuca* venom proteins using RP-HPLC **(A)**, followed by SDS-PAGE **(B)**. Two mg of venom were fractionated on a C_{18} column and eluted with an acetonitrile gradient (dashed line), followed by further separation of protein fractions by SDS-PAGE under reducing conditions. Molecular weight markers (M) are indicated in kDa. Coomassie-stained bands were excised, in-gel digested with trypsin, and subjected to MALDI-TOF/TOF analysis for assignment to protein families, as shown in Table 1.

Figure 3: Composition of the *N. melanoleuca* venom proteome according to protein families, expressed as percentages of total protein content based on w%. **3FTx**: three-finger toxins; **PLA_2**: phospholipase A_2s; **CRISP**: cysteine-rich secretory proteins; **NGF**: Nerve growth factors; **KUN**: Kunitz-type serine protease inhibitors; **OHA**: Ohanins/vespryns; **MP**: Snake venom metalloproteinases; **DRE**: DNA/RNA non-specific endonucelases; **PDE**: Endonucleases/Phosphodiesterases; **FMO**: Flavin monoamine oxidases; **GPF**: glutathione peroxidases; **CELF**: Type-B carboxylesterases/lipases; **SUSHI**: Selectins; **UNK**: Unknown.

Figure 4: (A) Comparison of the phospholipase A_2 activity of 20 µg of the venoms of *N. melanoleuca*, *N. mossambica*, *N. nigricollis*, and *Bothrops asper* on 4-nitro-3-octanoyloxybenzoic acid synthetic substrate. **(B)** Comparison of the proteolytic

activity of 20 µg of venoms of *N. melanoleuca*, *N. mossambica*, *N. nigricollis*, and *Bothrops asper* on azocasein substrate. Each bar represents mean ± SD of triplicates.

Figure 5: ELISA titrations of antivenoms against immobilized crude venoms of *N. melanoleuca*. **SAVP**: SAIMR Polyvalent Snake Antivenom from South African Vaccine Producers. **African**: Snake Venom Antiserum (African) from VINS Bioproducts Ltd. **Central Africa**: Snake Venom Antiserum (Central Africa) from VINS Bioproducts Ltd. **Control**: Normal horse serum. Each point represents mean ± SD of triplicate wells. Antivenom titrations are represented as volumetric dilutions in **(A)**, or as protein concentrations in **(B)**.

Figure 6: ELISA-based immunoprofiling of antivenoms against HPLC fractions of *N. melanoleuca* venom. For identification of venom fractions see Table 2. **SAVP**: SAIMR Polyvalent Snake Antivenom from South African Vaccine Producers. **African**: Snake Venom Antiserum (African) from VINS Bioproducts Ltd. **Central Africa**: Snake Venom Antiserum (Central Africa) from VINS Bioproducts Ltd. **Control**: Normal horse serum. Each bar represents mean ± SD of triplicate wells.

Table 1: Assignment of the RP-HPLC isolated fractions of *Naja melanoleuca* venom to protein families by MALDI-TOF-TOF of selected peptide ions from in-gel trypsin-digested protein bands.

Peak	%	Mass (kDa)▼	Peptide ion		MS/MS-derived sequence *	Conf (%)	Sc	Protein family **	Related protein; access code
			m/z	z					
3a	0.7	19	1466.7	1	KGIEINCCTTDR	99	8	3FTx	Alpha-neurotoxin NTX-1, *Naja sputatrix*; Q9YGJ6
			1338.6	1	GIEINCCTTDR	99	10		
3b	6.1	14	1229.5	1	TCPGETNCYK	99	7	3FTx	Short neurotoxin 1, *Naja melanoleuca*; P01424
			1039.4	1	INCCTTDR	99	8		
			1357.6	1	TCPGETNCYKK	99	10		
			1772.7	1	MECHNQQSSQPPTTK	99	18		
			1497.7	1	QWSDHRGTIIER	98.6	8		
			1427.5	1	INCCTTDRCNN	95.6	6		
3c	0.9	11	-	-	-	-	-	UNK	Unknown
4a	0.2	14	1338.6	1	GIEINCCTTDR	99	9	3FTx	Alpha-neurotoxin NTX-1, *Naja sputatrix*; Q9YGJ6
			1466.7	1	KGIEINCCTTDR	96.3	6		
4b	0.4	13	1772.7	1	MECHNQQSSQPPTTK	99	16	3FTx	Short neurotoxin 1, *Naja melanoleuca*; P01424
5a	0.3	15	1338.6	1	GIEINCCTTDR	99	9	3FTx	Alpha-neurotoxin NTX-1, *Naja sputatrix*; Q9YGJ6
5b	0.8	13	1772.7	1	MECHNQQSSQPPTTK	99	9	3FTx	Short neurotoxin 1, *Naja melanoleuca*; P01424
6	0.4	-	-	-	-	-	-	UNK	Unknown
7a	0.8	12	2365.1	1	YSRDNESSKTCPSGQ(N^{da})VCFK	97.3	10	3FTx	Short-chain 3FTx 7, *Bungarus flaviceps*; D5J9P4
7b	0.6	12	1316.5	1	TWCDNFCASR	99	6	3FTx	Long neurotoxin 2, *Naja melanoleuca*; P01388
			1628.6	1	CCSTDNCNPFPTR	94.4	6		
8a	3.2	12	1698.8	1	LTCLICPEKYCNK	99	9	3FTx	Weak toxin S4C11, *Naja melanoleuca*; P01400
			1317.6	1	GCAATCPEAKPR	99	12		
			1040.5	1	FYEGNLLGK	99	12		
			1196.6	1	FYEGNLLGKR	99	11		
			1137.5	1	NGENICFKR	99	12		
			1342.5	1	TWCDAWCTSR	99	7	3FTx	Long neurotoxin OH-55, *O. hannah*; Q53B58

8b	4.5	10	2472.0	1	CFITPDVTSQICADGHVCYTK	99	19	3FTx	Long neurotoxin 2, *Naja melanoleuca*; P01388
			1628.6	1	CCSTDNCNPFPTR	99	19		
			1342.5	1	TWCDAWCTSR	99	19	3FTx	Long neurotoxin OH-55, *O.hannah*; Q53B58
8c	5.5	10	2472.2	1	CFITPDVTSQICADGHVCYTK	99	20	3FTx	Long neurotoxin 2, *Naja melanoleuca*; P01388
			1628.7	1	CCSTDNCNPFPTR	99	19		
			1342.6	1	TWCDAWCTSR	99	15	3FTx	Long neurotoxin OH-55, *O. hannah*; Q53B58
9a	1.1	13	1298.6	1	TWCDAWCGSR	97	11	3FTx	Long neurotoxin OH-55, *O. hannah*; Q53B58
			2366.1	1	SIFGVTT(E^{na})DCPDGQNLCFKR	99	11	3FTx	Muscarinic toxin-like prot.2, *N. kaouthia*; P82463
9b	0.9	10	1342.6	1	TWCDAWCTSR	99	14	3FTx	Long neurotoxin OH-55, *O. hannah*; Q53B58
			2197.0	1	TPETTEICPDSWYFCYK	99	25	3FTx	Muscarinic toxin-like prot., *B. multicinctus*; Q9W727
10a	0.4	12	1298.6	1	TWCDAWCGSR	99	13	3FTx	Long neurotoxin OH-55, *O. hannah*; Q53B58
			1241.5	1	WCDAWCTSR	99	10		
10b	0.3	10	1342.6	1	TWCDAWCTSR	99	11	3FTx	Long neurotoxin OH-55, *O. hannah*; Q53B58
			1628.7	1	CCSTDNCNPFPTR	99	16		
			1508.7	1	ITCSAEETFCYK	99	15	3FTx	Bucandin, *Bungarus candidus*; P81782
10c	0.2	10	1342.6	1	TWCDAWCTSR	99	11	3FTx	Long neurotoxin OH-55 *O.hannah*; Q53B58
			1628.7	1	CCSTDNCNPFPTR	99	9		
			1361.6	1	YVCCNTDRCN	98.1	7	3FTx	Cytotoxin 1, *Naja melanoleuca*; P01448
			1508.7	1	ITCSAEETFCYK	99	14	3FTx	Bucandin, *Bungarus candidus*; P81782
11	0.6	10	2218.2	1	NLCYQMYMVSKSTIPVKR	98.2	6	3FTx	Cytotoxin 1, *Naja melanoleuca*; P01448
			1436.7	1	NLCYQMYMVSK	96.8	6		
12a	trace	14	1415.7	1	CKNPNPEPSGCR	99	12	NGF	Nerve growth factor, *Naja naja*; P01140
			1363.7	1	ALTMEGNQASWR	99	16		
			2261.0	1	GIDSSHWNSYCTETDTFIK	99	18		
			2768.5	1	TTATDIKGNTVTVMENVNLDNKVYK	97.5	6		
			1813.9	1	APYIDKNYNIDFNAR	99	16	PLA_2	PLA_2 DE-III, *Naja melanoleuca*; P00601
	trace		1864.8	1	SWWHFADYGCYCGR	99	16		

12b	3.8	10	1205.6	1	FKTIDECHR	99	8	KUN	Kunitz-type SP inhibitor 2, *Naja nivea*; P00986
			1424.7	1	FCELPAETGLCK	99	20		
	trace		1436.7	1	NLCYQMYMVSK	99	9	3FTx	Cytotoxin 1, *Naja melanoleuca*; P01448
13a	2.8	13	1818.9	1	ATYNDKNYNIDFNAR	99	19	PLA_2	PLA_2 1, *Naja melanoleuca*; P00599
			1241.6	1	NMIHCTVPNR	99	12		
			1126.6	1	NYNIDFNAR	99	12		
			1783.7	1	CCQIHDNCYGEAEK	99	10	PLA_2	PLA_2 DE-III, *Naja melanoleuca*; P00601
			1711.7	1	TYTYECSQGTLTCK	99	8		
			1864.8	1	SWWHFADYGCYCGR	99	18	PLA_2	PLA_2 4, *Naja sagittifera*; Q6T179
13b	2.0	10	1436.6	1	NLCYQMYMVSK	99	15	3FTx	Cytotoxin 1, *Naja melanoleuca*; P01448
			1361.5	1	YVCCNTDRCN	99	8		
14a	2.7	13	3350.4	1	TYTYESCQGTLTSCGAN(N^{da})KCAASVCDDR	99	8	PLA_2	PLA_2 DE-II, *Naja melanoleuca*; P00600
			1232.6	1	NMIQCTVPNR	99	12		
			2953.3	1	GGSGTPVDDLDRCCQIHDNCYGEAEK	96.8	7		
			1813.9	1	APYIDKNYNIDFNAR	99	24	PLA_2	PLA_2 DE-III, *Naja melanoleuca*; P00601
			1864.8	1	SWWHFADYGCYCGR	99	19		
14b	8.1	10	1361.5	1	YVCCNTDRCN	99	11	3FTx	Cytotoxin 1, *Naja melanoleuca*; P01448
			1436.6	1	NLCYQMYMVSK	99	17		
	trace		1813.9	1	APYIDKNYNIDFNAR	99	8	PLA_2	PLA_2 DE-III, *Naja melanoleuca*; P00601
15a	7.1	14	1123.6	1	ISGCWPYIK	99	9	PLA_2	PLA_2 DE-III, *Naja melanoleuca*; P00601
			2102.0	1	APYIDKNYNIDFNARCQ	99	9		
			1813.9	1	APYIDKNYNIDFNAR	99	25		
			2953.3	1	GGSGTPVDDLDRCCQIHDNCYGEAEK	98.7	7		
			1232.6	1	NMIQCTVPNR	99	12	PLA_2	PLA_2 muscarinic inhibitor, *Naja sputatrix*; Q92084
			1864.8	1	SWWHFADYGCYCGR	99	20		
15b	5.6	10	1988.9	1	SSLLVKYVCCNTDRCN	99	8	3FTx	Cytotoxin 1, *Naja melanoleuca*; P01448
			2832.4	1	TCPAGKNLCYQMYMVSKSTIPVKR	99	7		
			1361.5	1	YVCCNTDRCN	99	11		
			1436.6	1	NLCYQMYMVSK	99	18		

16a	0.4	14	1783.7	1	CCQIHDNCYGEAEK	99	12	PLA_2	PLA_2 DE-III, *Naja melanoleuca*; P00601
			1123.6	1	ISGCWPYIK	99	11		
			1813.9	1	APYIDKNYNIDFNAR	99	22		
			1232.6	1	NMIQCTVPNR	99	11	PLA_2	PLA_2 muscarinic inhibitor, *Naja sputatrix*; Q92084
			1050.4	1	DYGCYCGR	99	11		
			1864.8	1	SWWHFADYGCYCGR	99	20		
16b	1.3	10	1361.5	1	YVCCNTDRCN	99	11	3FTx	Cytotoxin 1, *Naja melanoleuca*; P01448
			1436.6	1	NLCYQMYMVSK	99	17		
17	3.4	11	1646.9	1	IKCHNTLLPFIYK	99	19	3FTx	Cytotoxin homolog 2, *Naja melanoleuca*; P01474
			1353.6	1	TCPEGQNLCFK	99	18		
			1405.7	1	CHNTLLPFIYK	99	17		
18	4.2	10	2125.1	1	TCPEGQNLCFKGTLKFPK	99	7	3FTx	Cytotoxin homolog 2, *Naja melanoleuca*; P01474
			1353.6	1	TCPEGQNLCFK	99	16		
			1646.9	1	IKCHNTLLPFIYK	99	18		
			1405.7	1	CHNTLLPFIYK	99	17		
19a	0.1	24	2768.4	1	TTATDIKGNTVTVMENVNLDNKVYK	99	6	NGF	Nerve growth factor, *Naja naja*; P01140
			1415.6	1	CKNPNPEPSGCR	99	10		
			2261.0	1	GIDSSHWNSYCTETDTFIK	99	21		
			1363.7	1	ALTMEGNQASWR	99	16		
19b	0.3	14	2768.4	1	TTATDIKGNTVTVMENVNLDNKVYK	99	11	NGF	Nerve growth factor, *Naja naja*; P01140
			1415.6	1	CKNPNPEPSGCR	99	12		
			2261.0	1	GIDSSHWNSYCTETDTFIK	99	27		
			1363.7	1	ALTMEGNQASWR	99	17		
			1824.0	1	FIRIDTACVCVITKK	97.7	8		
	trace		1864.7	1	SWWHFADYGCYCGR	99	12	PLA_2	PLA_2 B, *Naja sputatrix*; Q92085
19c	0.4	11	3013.3	1	FTCFTTPSDTSETCPDGQNICYEKR	99	9	3FTx	Weak toxin CM-2, *Naja haje*; P01415
			2857.1	1	FTCFTTPSDTSETCPDGQNICYEK	66.8	8		
20a	0.5	14	2270.1	1	ADVTFDSNTAFESLVVSPDKK	99	20	OHA	Thaicobrin, *Naja kaouthia*; P82885
			2595.3	1	SGKHFFEVKYGTQREWAVGLAGK	99	10		
			3286.6	1	TVENVGVSQVAPDNPERFDGSPCVLGSPGFR	99	13		
			1535.8	1	YGTQREWAVGLAGK	99	17		
			1810.9	1	TVENVGVSQVAPDNPER	99	24		
20b	1.0	11	3013.3	1	FTCFTTPSDTSETCPDGQNICYEKR	99	9	3FTx	Weak toxin CM-2, *Naja haje*; P01415

			2857.1	1	FTCFTTPSDTSETCPDGQNICYEK	99	11		
21a	0.3	31	1441.7	1	WSNLP(T^{dh})LCQGKR	99	12	SUSHI	C' decay-accelerating factor, *O. hannah*; ETE59511
21b	4.5	11	3013.3	1	FTCFTTPSDTSETCPDGQNICYEKR	99	11	3FTx	Weak toxin CM-2, *Naja haje*; P01415
			2857.2	1	FTCFTTPSDTSETCPDGQNICYEK	99	12		
22	0.6	15	1424.8	1	KKYIWEWTDR	99	11	CTL	CTL LP-Pse-6, *Pseudonaja modesta*; R4G314
			1168.6	1	YIWEWTDR	99	10		
23	0.8	23	1349.8	1	QIVDKHNALRR	99	9	CRISP	CRISP, *Micropechis ikaheka*; A0A024AX20
			1849.9	1	NMLQMEWNSNAAQNAK	99	16		
			2006.0	1	NMLQMEWNSNAAQNAKR	99	17		
			1031.4	1	CAASCFCR	95.2	8		
			1826.9	1	WADRCSFAHSPSHLR	99	7	CRISP	Latisemin, *Laticauda semifasciata*; Q8JI38
			1298.6	1	CSFAHSPSHLR	99	16		
24a	0.9	84	1890.8	1	LQPHAQCDSEECCEK	99	11	MP	Atrase-A, *Naja atra*; D5LMJ3
			1087.6	1	EHQEYLLR	99	10		SVMP-Aca-4, *Acanthophis wellsi*; R4G2D3
			1497.8	1	ERPQCILNKPSR	99	13		
			1388.7	1	YIEFYVVVDNK	99	13		
24b	0.4	24	1298.7	1	CSFAHSPSHLR	99	8	CRISP	Latisemin, *Laticauda semifasciata*; Q8JI38
									Natrin-1, *Naja atra*; Q7T1K6
			2014.0	1	VLEGIQCGESIYMSSNAR	99	10		
25	6.4	24	1451.8	1	QKEIVDLHNSLR	99	13	CRISP	Ophanin, *Ophiophagus hannah*; Q7ZT98
			1168.6	1	NVDFNSESTR	99	12		
26a	1.5	58	1720.8	1	AAKDDCDLPELCTGR	99	14	MP	SVMP 1, *Micrurus fulvius*; U3EPC7
			1416.7	1	YIEFYVVVDNR	99	12		
			1544.8	1	KYIEFYVVVDNR	99	14		
								MP	SVMP kaouthiagin, *Naja kaouthia*; P82942
			1471.7	1	TAPAFQFSSCSIR	99	17		
26b	0.5	44	1720.8	1	AAKDDCDLPELCTGR	99	15	MP	SVMP 1, *Micrurus fulvius*; U3EPC7
			1544.9	1	KYIEFYVVVDNR	94.2	6		
			1471.7	1	TAPAFQFSSCSIR	99	17	MP	Atrase-B, *Naja atra*; D6PXE8
26c	0.3	29	2110.1	1	GCQQTFAVVGAVPGDTYIAR	99	24	DRE	Endonucl.domain-containing 1 prot *O. hannah*;
			1634.8	1	GHLNPNGHQPDYSAK	99	16		V8N4Y2

			2243.0	1	LNGGAWNNYEQTTMQQMTR	99	13		
			2024.0	1	EVVDSFQDHCPQFFLR	99	19	DRE	Endonucl.domain-containing 1 prot *M. fulvius*; U3FCT9
27a	1.2	73	1087.6	1	EHQEYLLR	99	12	MP	Atrase-A, *Naja atra*; D5LMJ3
			1646.8	1	VYE(M[ca])VNALNTMYR	98.1	7	MP	Mocarhagin, *Naja mossambica*; Q10749
27b	0.7	54	1720.8	1	AAKDDCDLPELCTGR	99	8	MP	SVMP 1, *Micrurus fulvius*; U3EPC7
			1416.7	1	YIEFYVVVDNR	99	13		
			1544.8	1	KYIEFYVVVDNR	99	13		
			1087.6	1	EHQEYLLR	99	6	MP	Atrase-A, *Naja atra*; D5LMJ3
27c	0.2	49	1416.8	1	YIEFYVVVDNR	99	11	MP	MTP4, *Micrurus fulvius*; U3FWL3
			1544.8	1	KYIEFYVVVDNR	99	12		
			1087.6	1	EHQEYLLR	99	9	MP	Atrase-A, *Naja atra*; D5LMJ3
27d	0.3	41	1720.8	1	AAKDDCDLPELCTGR	99	10	MP	SVMP 1, *Micrurus fulvius*; U3EPC7
			1416.8	1	YIEFYVVVDNR	99	12		
			1544.9	1	KYIEFYVVVDNR	98.3	9		
			1087.6	1	EHQEYLLR	99	9	MP	Atrase-A, *Naja atra*; D5LMJ3
28a	1.1	118	1518.8	1	LWNYFHSTLLPK	99	9	PDE	Phosphodiesterase 1, *Micrurus fulvius*; U3FAB3
			2106.0	1	RPDFSTLYIEEPDTTGHK	99	17		
28b	0.2	73	1087.6	1	EHQEYLLR	99	6	MP	Atrase-A, *Naja atra*; D5LMJ3
29a	0.2	71	1224.7	1	AIAEMVHLNAR	98.9	7	FMO	L-amino-acid oxidase, *Naja atra*; A8QL58
29b	0.2	30	1329.7	1	IHDIKWNFEK	99	13	GPF	Glutathione peroxidase, *O.hannah*; V8P395
			1492.8	1	LVILGFPCNQFGK	99	8		
			1385.8	1	FLVNPQGKPVMR	99	12		
29c	0.7	25	1492.8	1	LVILGFPCNQFGK	99	12	GPF	Glutathione peroxidase, *O.hannah*; V8P395
			1385.8	1	FLVNPQGKPVMR	99	11		
30a	0.2	250	1344.7	1	KFWEADGIHGGK	99	9	FMO	L-amino-acid oxidase, *Naja atra*; A8QL58
			1224.7	1	AIAEMVHLNAR	99	12		

30b	0.6	74	1539.8	1	NNKVYAYLFDHR	99	8	CELF	ACN-Den-1, *Denisonia devisi*; R4FJM1
			2390.2	1	AVTIFGESAGAASVGMHLLSTQSR	99	10		
			2137.1	1	AILQSGAPNAPWATVTPAESR	99	18	CELF	Acetylcholinesterase 1, *O.aestivus*; A0A098LYD4
			1017.6	1	VATQTGWVR	99	8	CELF	Acetylcholinesterase 2, *Python regius*; A0A098LWS5
30c	1.0	62	1224.7	1	AIAEMVHLNAR	99	15	FMO	L-amino-acid oxidase, *Naja atra*; A8QL58
			2546.2	1	SPLEECF(R^{ca})EADYEEFLEIAR	99	13	FMO	L-amino-acid oxidase, *B.multicinctus*; A8QL51
31a	0.6	76	2137.1	1	AILQSGAPNAPWATVTPAESR	82.7	5	CELF	Acetylcholinesterase 1, *Echis coloratus*; A0A0A1WDT1
31b	0.5	68	1216.6	1	FWEADGIHGGK	99	8	FMO	L-amino-acid oxidase, *Naja atra*; A8QL58
			1344.7	1	KFWEADGIHGGK	99	7		
			1224.7	1	AIAEMVHLNAR	99	13		
31c	0.6	62	1935.0	1	TLSYVTADYVIVCSTSR	99	8	FMO	LAO-Hop-2, *H.bungaroides*; R4FK16
			1344.7	1	KFWEADGIHGGK	99	6		
32a	0.9	64	2517.2	1	STSMVAITMAHQMGHNLGMNDDR	99	7	MP	Mocarhagin, *Naja mossambica*; Q10749
			1754.8	1	AAK(N^{da})DCDFPELCTGR	99	15		
			1010.5	1	CPTDSFQR	99	9	MP	SVMP 1, *Micrurus fulvius*; U3EPC7
			1206.7	1	(N^{da})VDAFLGKWR	98.7	9	FABP	Fatty acid-binding protein, *M. fulvius*; U3FZI3
32b	0.5	56	1754.8	1	AAKNDCDFPELCTGR	99	12	MP	Mocarhagin, *Naja mossambica*; Q10749
			1010.5	1	CPTDSFQR	99	7	MP	SVMP 1, *Micrurus fulvius*; U3EPC7
			1087.6	1	EHQEYLLR	99	8	MP	Atrase-A, *Naja atra*; D5LMJ3
33	2.8	52	1476.8	1	CPIMTNQCIALR	99	13	MP	Atragin, *Naja atra*; D3TTC2
			1140.6	1	DSCFTLNQR	99	11		
			1915.1	1	CPIMTNQCIALRGPGVK	98.3	7		
			1687.9	1	RTKPAY(Q^{da})FSSCSVR	95.4	9		
			1087.6	1	EHQEYLLR	99	12	MP	Atrase-A, *Naja atra*; D5LMJ3

* Cysteine residues are carbamidomethylated. Confidence (Conf) and Score (Sc) values are calculated by the Paragon algorithm of ProteinPilot®. ▼: reduced SDS-PAGE mass estimations, in kDa. Possible, although unconfirmed/ambiguous amino acid modifications suggested by the automated identification software are shown in parentheses, with the following abbreviations: [da]: deamidated; [na]: Na cation; [dh]: dehydrated; [ca]: carbamylated.

** Protein family abbreviations: 3FTx: Three-finger toxin; PLA_2: Phospholipase A_2; CRISP: Cysteine-rich secretory protein; NGF: Nerve growth factor; KUN: Kunitz-type serine protease inhibitor; OHA: Ohanin/vespryn; MP: Metalloproteinase; DRE: DNA/RNA non sp-endonuclease; CTL: C-type lectin; PDE: Endonuclease/Phosphodiesterase; FMO: Flavin monoamine oxidase; GPF: Glutathione peroxidase; CELF: Type-B carboxylesterase/lipase; SUSHI: Selectins – cell adhesion (http://pfam.xfam.org/family/PF00084); UNK: Unknown.

Table 2
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Table 2: Lethality and Toxicity Score of RP-HPLC fractions of the venom of *N. melanoleuca*

Peak	w%	mol%	Protein family	LD_{50} (95% conf. limits)	Reported LD_{50} (mg/kg)	Toxicity score w% / LD_{50} (kg/mg)[1]	Molecular Toxicity score mol% / LD_{50} (kg/mg)[1]
Whole venom	100	100		0.66 (0.49-0.92)	0.289*	151	151
3	7.7	10.2	**3FTx** Alpha-neurotoxin NTX-1 *Naja sputatrix*; Q9YGJ6 Short neurotoxin 1 *Naja melanoleuca*; P01424	0.13 (0.06-0.34)		59.2	78.8
4	0.6	0.9	**3FTx** Alpha-neurotoxin NTX-1 *Naja sputatrix*; Q9YGJ6 Short neurotoxin 1 *Naja melanoleuca*; P01424	0.13 (0.05-0.33)		4.6	6.9
5	1.1	1.6	**3FTx** Alpha-neurotoxin NTX-1 *Naja sputatrix*; Q9YGJ6 Short neurotoxin 1 *Naja melanoleuca*; P01424	0.11 (0.03-0.23)		10	15.0
7	1.4	1.9	**3FTx** Short-chain 3FTx 7 *Bungarus flaviceps*;D5J9P4 Long neurotoxin 2 *Naja melanoleuca*;P01388	>0.53		<2.6	<3.6
8	13.2	17.3	**3FTx** Weak toxin S4C11 *Naja melanoleuca*; P01400 Long neurotoxin OH-55 *O.hannah*; Q53B58 Long neurotoxin 2 *Naja melanoleuca*; P01388	0.15 (0.06-0.42)	>20 (P01400) [9]	88	115.5
9	2.0	2.7	**3FTx** 3FTx-Den-15 *Denisonia devisi*; R4FID4 Muscarinic toxin-like prot.2 *N. kaouthia*; P82463 Long neurotoxin OH-55 *O.hannah*; Q53B58 Muscarinic toxin-like prot *B.multicinctus*; Q9W727	>0.53		<3.8	<5.0

10	0.9	1.2	**3FTx** 3FTx-Den-15 *Denisonia devisi*; R4FID4 3FTx-Ech-35 *Echiopsis curta*; R4G7H1 Long neurotoxin OH-55 *O.hannah*; Q53B58 Bucandin *Bungarus candidus*; P81782 Cytotoxin 1 *Naja melanoleuca*; P01448	>0.53	1.36 (P01448) [11]	<1.7	<2.2
11	0.6	0.9	**3FTx** Cytotoxin 1 *Naja melanoleuca*; P01448	>0.53	1.36 (P01448) [11]	<1.1	<1.7
12	3.8	6.0	Kunitz-type SP inhibitor 2 *Naja nivea*; P00986	>0.53		<7.2	<11.4
13	4.8 (1:1 mix)	5.2 (1:1 mix)	**PLA_2** PLA_2 1 *Naja melanoleuca*; P00599 PLA_2 DE-III *Naja melanoleuca*; P00601 PLA_2 4 *Naja sagittifera*; Q6T179 **3FTx** Cytotoxin 1 *Naja melanoleuca*; P01448	>0.53	1.36 (P01448) [11]	<9.1	<9.9
14	10.8 (1:3 mix)	8.3 (1:3 mix)	**PLA_2** PLA_2 DE-II *Naja melanoleuca*; P00600 PLA_2 DE-III *Naja melanoleuca*; P00601 **3FTx** Cytotoxin 1 *Naja melanoleuca*; P01448	>0.53	1.36 (P01448) [11]	<20.4	<15.6
15	12.7 (1:1 mix)	14.0 (1:1 mix)	**PLA_2** PLA_2 DE-III *Naja melanoleuca*; P00601 PLA_2 muscarinic inhibitor *Naja sputatrix*; Q92084 **3FTx** Cytotoxin 1 *Naja melanoleuca*; P01448	>0.53	1.36 (P01448) [11]	<24.0	<26.5
16	1.7 (1:3 mix)	2.3 (1:3 mix)	**PLA_2** PLA_2 DE-III *Naja melanoleuca*; P00601 PLA_2 muscarinic inhibitor *Naja sputatrix*; Q92084 **3FTx** Cytotoxin 1 *Naja melanoleuca*; P01448	>0.53		<3.2	<4.4

17	3.4	5.1	**3FTx** Cytotoxin homolog 2 *Naja melanoleuca*; P01474	>0.53		<6.4	<9.6
18	4.2	6.3	**3FTx** Cytotoxin homolog 2 *Naja melanoleuca*; P01474	>0.53		<7.9	<11.8
19	0.7 (1:1 mix)	0.8 (1:1 mix)	**NGF** Nerve growth factor *Naja naja*; P01140 Nerve growth factor *Naja naja*; P01140 **3FTx** Weak toxin CM-1c *H.haemachatus*; P25676	>0.53	54 (P25676) [38]	<1.3	<1.5
20	1.5 (1:2 mix)	1.9 (1:2 mix)	**OHA** Thaicobrin *Naja kaouthia*; P82885 **3FTx** Weak toxin CM-1c *H.haemachatus*; P25676	>0.53	54 (P25676) [38]	<2.8	<3.6
21	4.8	6.7	**SUSHI** C' decay-accelerating factor *O.hannah*; ETE59511 C'-decay-accelerating factor *O.hannah*; V8NC63 Weak toxin CM-2 *Naja haje*; P01415	>0.53		<9.1	<12.7
22	0.6	0.4	**CTL** CTL LP-Pse-6 *Pseudonaja modesta*; R4G314	>0.53		<1.1	<0.7

[1]Toxicity Score is defined as the ratio of protein fraction abundance (%) in the venom to its estimated median lethal dose (LD_{50}), whereas Molecular Toxicity Score corresponds to the ratio of protein abundance in molar terms (%) to LD_{50}. In the case of crude venom, the % abundance was 100%.

* : http://snakedatabase.org/pages/LD50.php#legendAndDefinitions

Figure 1
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Figure 2

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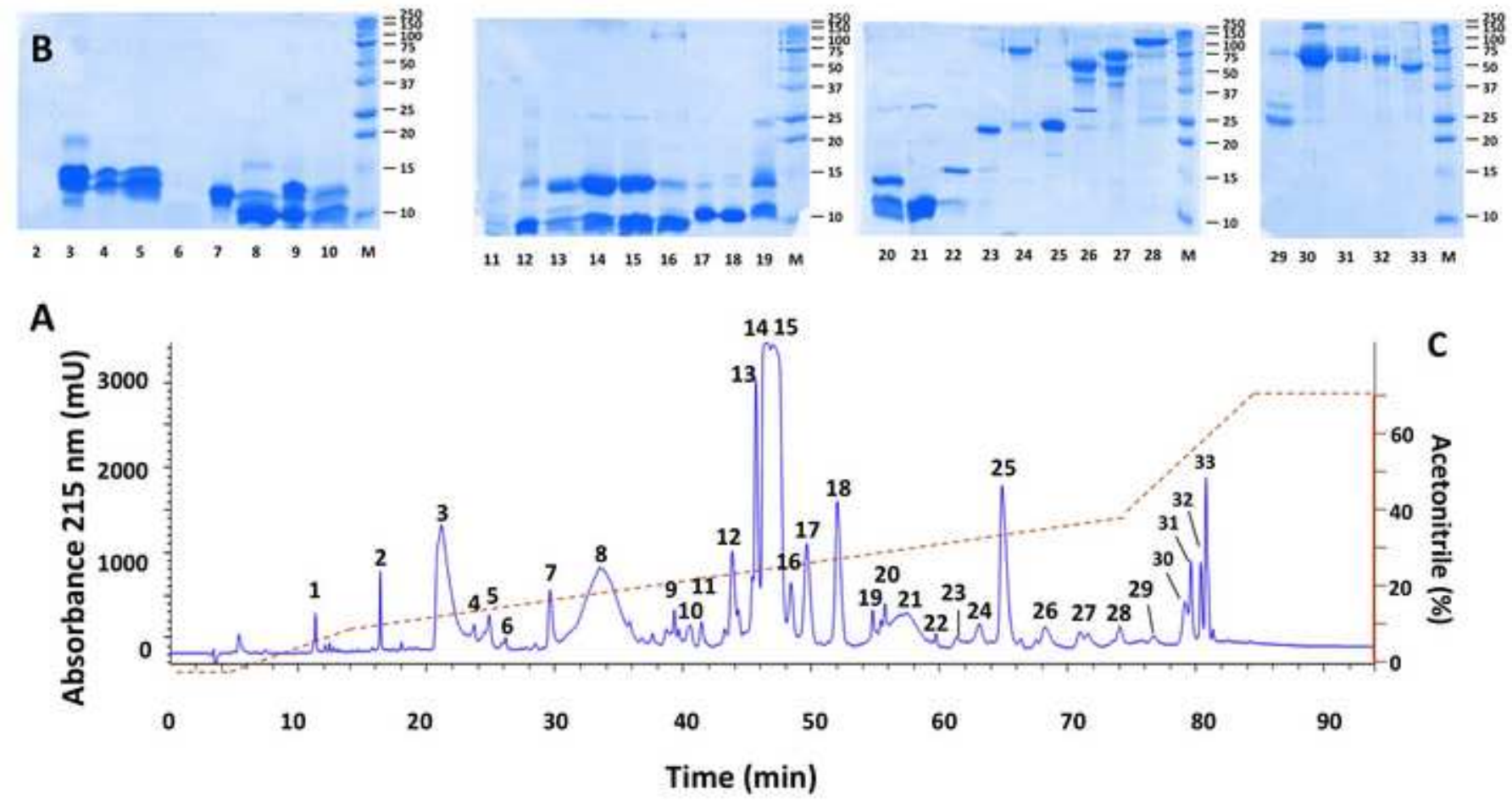

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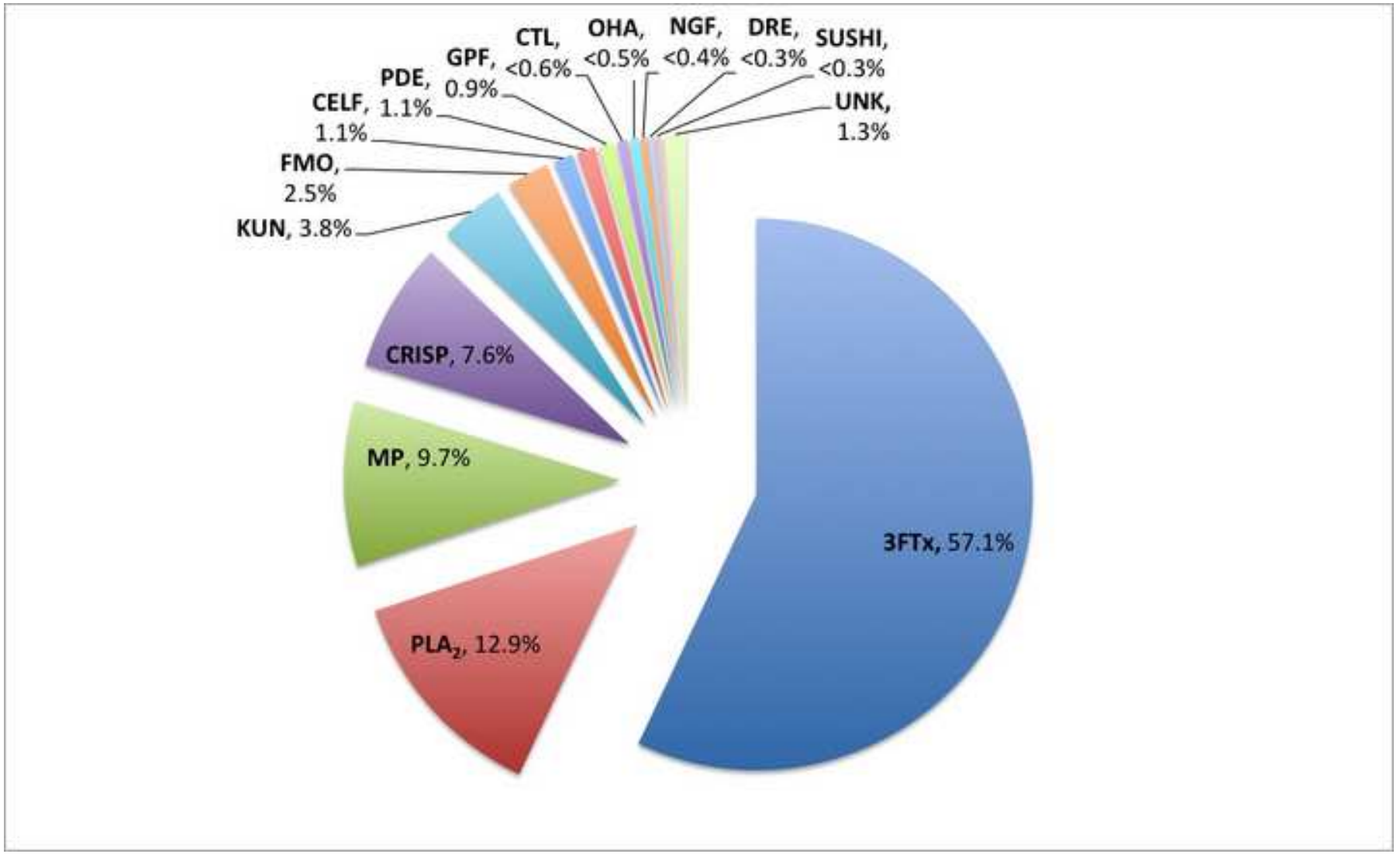

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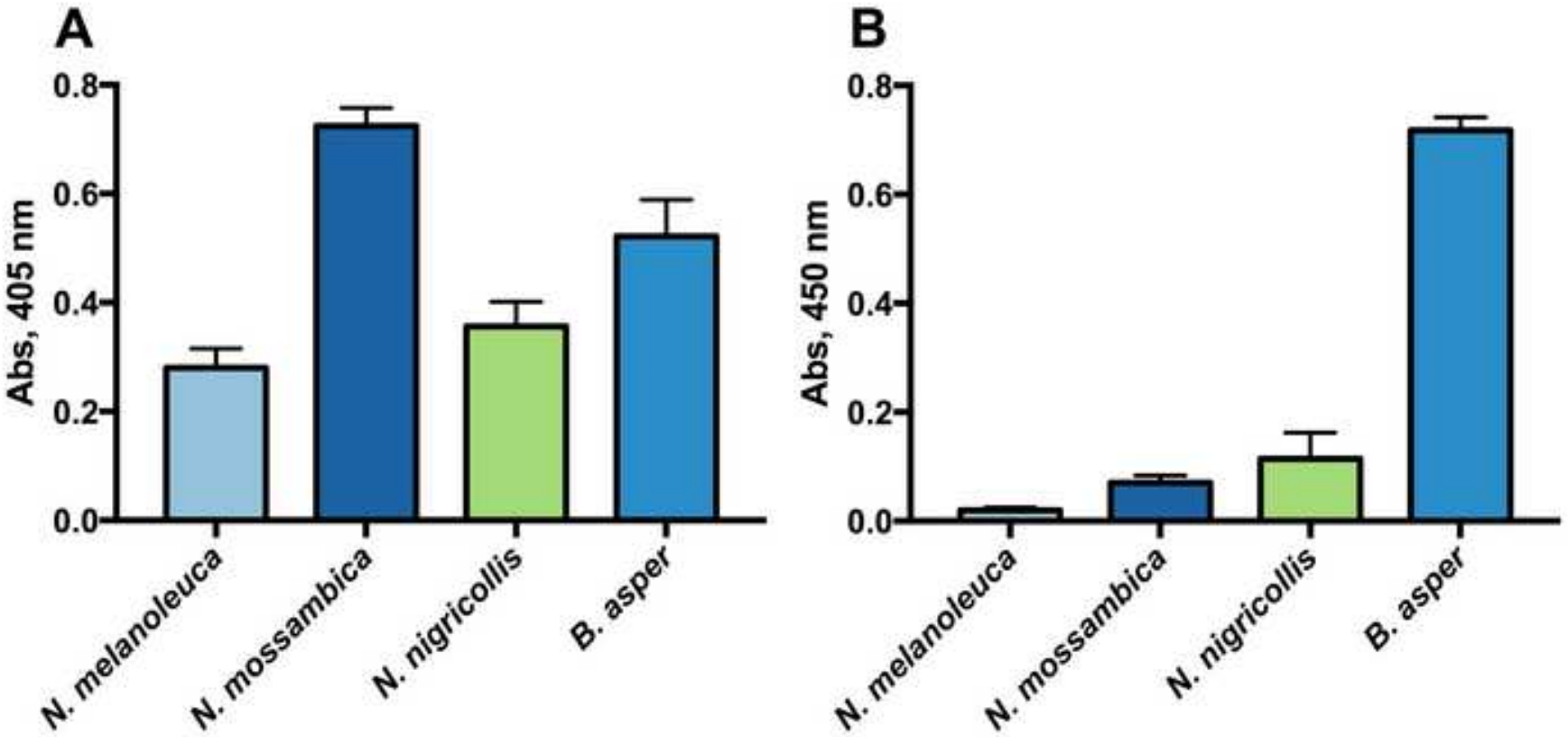

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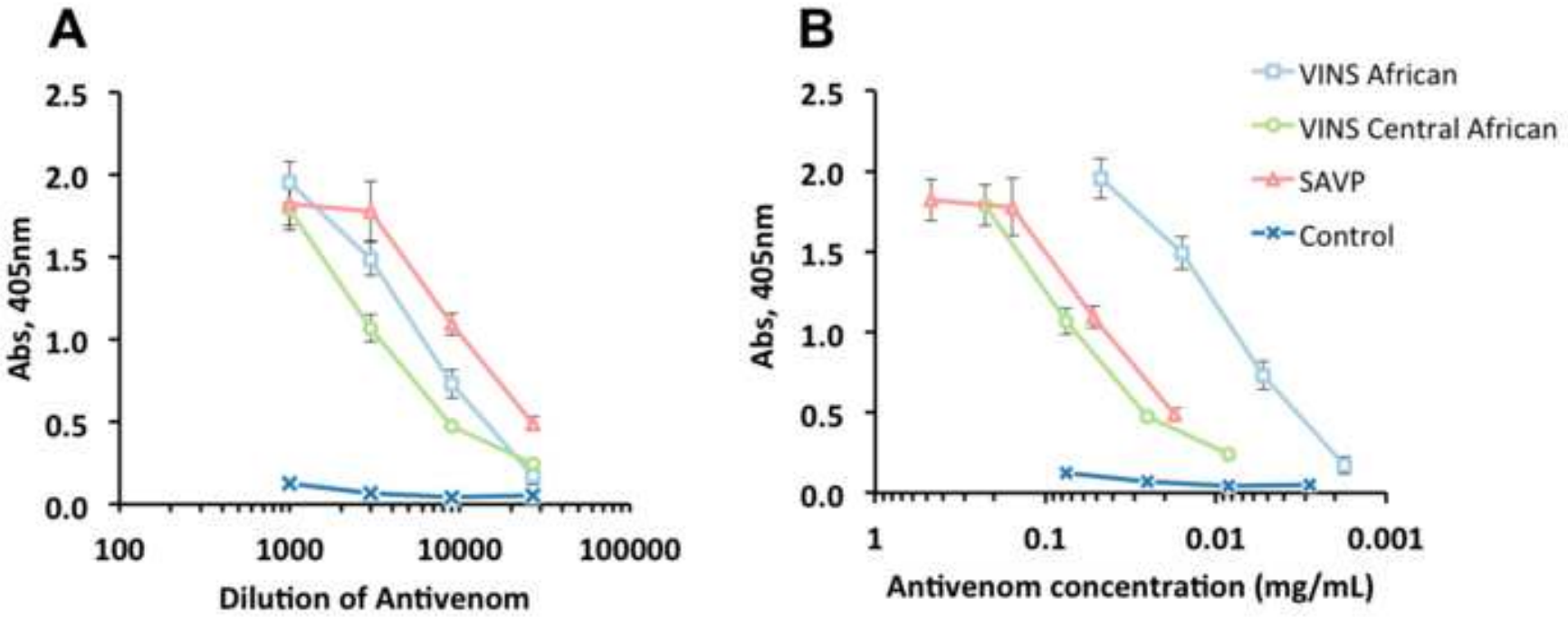

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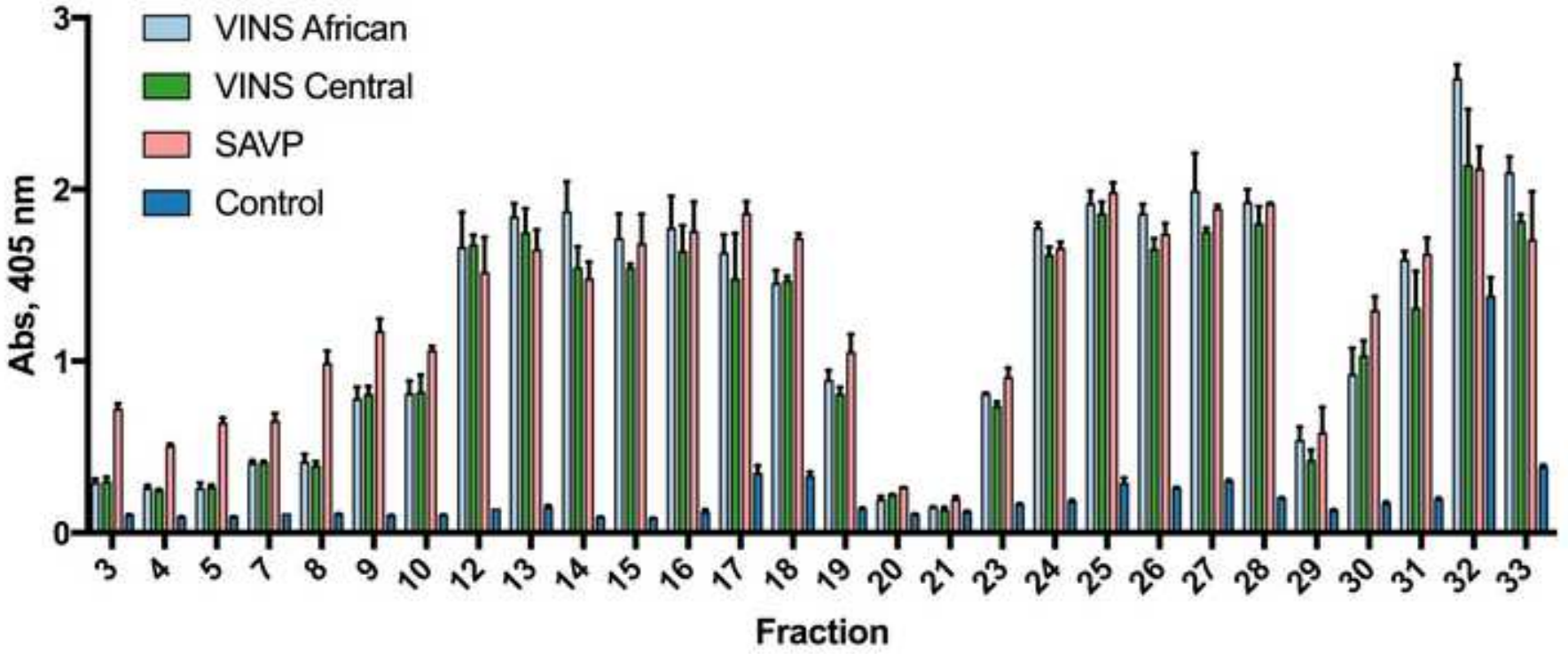

***Conflict of Interest**

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